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Molecular characterization and evidencing of the porcine *CRH* gene as a functional-positional candidate for growth and body composition

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Abstract

Corticotropin-releasing hormone (CRH), a major regulator of neuroendocrine response to stress, is involved in the control of energy balance and thus may affect body composition and growth. The porcine *CRH* (p*CRH*) gene was studied as a comparative-positional candidate for QTL for longissimus muscle area, average backfat thickness, carcass length, and average daily gain on test on porcine chromosome 4. Sequence of the complete transcriptional unit of p*CRH* gene spanning 2068 bp was determined along with 582 bp of the 5′-flanking region. Cross-species sequence comparison revealed a number of potential regulatory regions including an intronic evolutionary conserved region and an adjacent CpG island that may control cell-type specific expression of the *CRH* gene. A SNP in exon 2 (c.+83G > A) leading to a non-conservative amino acid exchange (p.28Arg > Gln) in the prohormone was identified that is segregating in the DUMI resource population. Linkage and association analysis based on this SNP revealed that for all four traits the p*CRH* gene falls in the QTL peak area and that the c.+83G > A SNP shows a highly significant additive effect ($p < 0.0001$). Physical mapping using the IMpRH panel assigned the p*CRH* gene to interval SW724-S0107, promoting the gene as a positional candidate also for QTL identified in other porcine resource populations. Additional four variable sites were identified that segregate in commercial pig breeds. Particularly interesting is a SNP (g.233C > T) in the 5′-flanking region that occurred in an evolutionary conserved motif. The knowledge of the DNA-variation of p*CRH* gene will facilitate follow-up studies necessary to provide definite genetic evidence of the effect of p*CRH* gene on body composition and growth.

Keywords: Body composition; Comparative genomics; CRH; Growth; Pig; Quantitative trait locus; Single nucleotide polymorphisms

Corticotropin-releasing hormone (CRH), a 41-amino acid neuropeptide, is a key coordinator of stress responses, including activation of the hypothalamo–pituitary–adrenal (HPA) and sympathoadrenal (SA) systems coupled with release of stress-responsive hormones glucocorticoids (in the pig cortisol) and catecholamines (adrenaline and noradrenaline), respectively [1]. One of the main functions of the stress-responsive hormones is mobilization of energy required for coping with stress by stimulating hepatic gluconeogenesis and influencing fat and protein metabolism in peripheral (e.g., adipose and muscle) tissue [2]. Selection for lower adiposity in pigs led to morphological and functional changes in the HPA axis. Overall, cortisol is associated with decreased growth and lean content in the pig and its level is generally higher in pig breeds with lower performance [3]. In contrast, catecholamines promote lean tissue deposition as evidenced by treatment of pigs with β-agonists—synthetic catecholamine analogs [2]. The function of CRH as a regulator of stress-hormone secretion implicates involvement of CRH in the control of energy homeostasis and consequently body composition and growth. Indeed, chronic hypersecretion of glucocorticoids in *CRH*

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transgenic mice leads to obesity, muscle wasting, and decreased linear growth [4]. On the other hand, activation of the SA system in obese (fa/fa) rats through central administration of CRH prevented their excessive weight gain [5]. Furthermore, CRH possesses a potent anorexigenic effect conserved among vertebrates including the pig [6], acting as a downstream mediator of leptin and melanocortin in the regulation of food intake [7,8]. On top of the functional evidences candidate gene studies in humans and cattle have shown that sequence variation of *CRH* gene is associated with variation in fat deposition [9], growth, and muscularity [10]. Recently quantitative trait loci (QTL) mapping in a porcine resource population, DUMI, indicated that *CRH* gene might affect body composition and growth in pigs as well. QTL affecting longissimus muscle area (LMA), average backfat thickness (BFT), carcass length (CL), and average daily gain on test (ADG) were mapped to central part of porcine chromosome 4 that was subsequently demonstrated to be orthologous to region q12.3–q21.13 of human chromosome 8 containing the *CRH* gene [11,12]. These convincing functional and genetical evidences promote the *CRH* gene as the primary comparative-positional candidate for the QTLs.

We aimed to finish and characterize the sequence of p*CRH* gene including the 5′-flanking region and to perform a DNA-variation survey. By linkage and physical mapping we confirmed the p*CRH* gene as a positional candidate not only for the QTL identified in the DUMI population but also for growth and body composition QTL on Ssc4 identified in other porcine resource populations. Analysis of a non-synonymous coding SNP segregating in the DUMI population revealed highly significant association with LMA, BFT, CL, and ADG, and provides a supporting evidence for the p*CRH* gene as a QTL affecting growth and body composition.

Materials and methods

Molecular characterization of the pCRH gene. The published partial cDNA sequence (Y15159) was completed by performing 5′- and 3′-rapid amplification of cDNA ends (RACE). RNA was isolated from available tissues that were reported to express *CRH* gene in humans and rodents, namely hypothalamus (Pietrain), fetal brain (Berlin Miniature Pig), pituitary (Duroc), and uterus (Pietrain), using TRI Reagent (Sigma, Taufkirchen, Germany) and subsequently pooled. For 5′-RACE the SMART™ RACE cDNA Amplification Kit (Clontech, Heidelberg, Germany) was employed. For 3′-RACE the cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and a T7-tailed oligo $d(T)_{13}$. To elucidate the genomic structure of p*CRH* gene, primers were designed in the presumed exons 1 and 2, and used to clone the intron sequence. In order to obtain the 5′-flanking region, a heterologous forward primer was designed based on the alignment of human, sheep, rat, and mouse sequences, and combined with a reverse primer annealing in the intron. Primers and PCR conditions used to obtain the p*CRH* gene sequence are listed in Table 1.

In order to identify DNA-variation suitable for analysis of p*CRH* gene as a positional candidate for QTL on Ssc4, a segment of exon 2 (g.1589–g.2216) was comparatively sequenced in grandparents of DUMI population. To discover DNA variation suitable for follow-up studies, three overlapping fragments spanning the 5′-flanking region, exon 1, intron, whole coding sequence, and a part of 3′-UTR (g.1–g.2216) were comparatively sequenced using a DNA panel of divergent breeds (German Landrace, Large White, Pietrain, Berlin Miniature Pig). In addition, 5′-flanking region (g.1–g.912) and exon 2 (g.1589–g.2216) segments were comparatively sequenced in each two German Landrace with high/low poststress cortisol level respectively. Sequencing of 3′-RACE fragments already revealed a number of variable sites in the 3′-UTR, therefore no additional polymorphism screening was performed for this region. Primers and PCR conditions used for comparative sequencing are given in Table 2. The PCR products were gel purified and either cloned in pGEM-T (Promega, Mannheim, Germany) or pCR2.1 (Invitrogen) vector and sequenced using the SequiTherm EXCEL Sequencing Kit (Biozym, Hess. Oldendorf, Germany) on a Licor 4200 automated sequencer or directly sequenced on a Megabace 750 capillary sequencer using Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany). Identified variable sites were described according to current nomenclature recommended by Human Genome Variation Society (http://www.genomic.unimelb.edu.au/mdi/mutnomen/).

In silico sequence analyses. Phylogenetic footprinting and identification of conserved/aligned transcription factor binding sites were performed using the Mulan [13] and zPicture/rVista2.0 [14] tools, respectively, which are accessible at Comparative Genomics Center (http://www.dcode.org). We defined segments with pairwise similarity of at least 75% over at least 50 bp and shared among all five species as evolutionary conserved regions (ECRs). *CRH* loci sequences from human (May 2004 assembly), chimpanzee (November 2003 assembly), dog (July 2004 assembly), cattle

Table 1
Primers and amplification conditions used for gene identification and DNA-variation survey

Target region	Primer sequence 5′–3′	Size (bp)	Ta (°C)
5′-End	UPM Oligo, SMART™ RACE Kit	403[a]	55
(c.-158–c.+245)	Rev: GCAGCGGGACTCTTATTGAG	gi[b]	
3′-End	Fwd: AGCAACAGGAAACTGATGGAG	555[a]	60
(c.+541–c.*519)	Rev: ACGACTCACTATAGGGCT$(T)_{13}$	gi[b]	
Intron	Fwd: CACCTACAGAAGCGCCTCAG	1076	54
(c.-37–c.+245)	Rev: GCAGCGGGACTCTTATTGAG	gi[b]	
5′-Flanking region	Fwd: CTTCATTCTTTTCCAGGCAG	921	55
(g.1–g.921)	Rev: GAATCTTGAGGCACAGTCAGC	gi,vs[b]	
Exon 1- intron- exon 2	Fwd: AACCCTTGAAAACCCCAGAGAC	1129	56
(g.672–g.1800)	Fwd[c]: GCTGACTGTGCCTCAAGATTC		
	Rev: GCAGCGGGACTCTTATTGAG	vs[b]	
Exon 2	Fwd: CTGTTGGTGGCTCTCTTGC	625	55
(g.1589–g.2216)	Rev: CAGCGCTATGGTACAGAATACTG	vs[b]	

[a] Length of the longest obtained segment.
[b] gi, primer pair used for gene identification; vs, primer pair used for DNA-variation survey.
[c] Internal oligo used for sequencing.

(September 2004 assembly), rat (June 2003 assembly), and mouse (March 2005 assembly) were retrieved from the UCSC website (http://genome.ucsc.edu). Partial feline sequences were retrieved from the National Center for Biotechnology Information (NCBI) Trace database (http://www.ncbi.nih.gov/Traces/trace.cgi) and assembled using CAP3 (http://bio.ifom-firc.it/ASSEMBLY/assemble.html). Ovine *CRH* gene and protein sequence (M22853) and protein sequences of human (NP_000747), dog (BAD10983) cattle (AAK83231), rat (AAA40965) and mouse (AAN07905) were retrieved at the NCBI website. Pyrimidine content of a 50-bp sliding window was computed using simple plot option at the Sequence Manipulation Suite accessible at http://bioinformatics.org/ and visualized in Excel. CpG island was identified using the CpG plot at the EBI website (http://www.ebi.ac.uk/emboss/cpgplot/).

Genotyping. For discrimination of allelic variants at positions g.233 and c.+83 Single Strand Conformation Polymorphism (SSCP) assays were developed (Fig. 5). SSCP analysis of the g.233 C > T SNP was performed on 12% (49:1 AA:Bis) native PAA gel run on a Macrophor apparatus (Pharmacia Biotech, Uppsala, Sweden) at 600 V for 2 h at 5 °C and subsequently 12 h at 18 °C and finally silver stained. Genotyping of c.+83G > A was performed as described previously [15]. The Simple Sequence Length Polymorphism (SSLP) at c.*44 was resolved on denaturing 7 % LongRanger (Biozym, Hess. Oldendorf, Germany) gels run on a Licor 4000 automated sequencer (Fig. 5). For genotyping of the c.-15+8 G > T and c.*151C > G SNP Hin1I and RsaI PCR-restriction fragment length polymorphism (PCR-RFLP), respectively, were designed (Fig. 4). PCR fragments were digested with 10 U of the respective enzyme overnight at conditions recommended by manufacturer (MBI Fermentas, St. Leon-Rot, Germany) and the restriction fragments (c.-15 + 8 G > T: 168 bp + 82 bp/250 bp; c.*151C > G: 303 bp + 109 bp/224 bp + 109 bp + 79 bp) resolved on 3% agarose gels. Primers used to amplify the five variable sites are listed in Table 2.

Physical mapping. Physical mapping was performed using the INRA—University of Minnesota porcine Radiation Hybrid (IMpRH) panel [16]. The panel was screened by a stepdown PCR (Table 2) and the products were resolved on 2% agarose gels. Regional assignment was obtained using multipoint analysis option of the IMpRH mapping tool at the IMpRH server (http://www.toulouse.inra.fr).

Statistic analysis. The animals used for association and QTL analysis were from a three-generation porcine F2 resource population (DUMI) based on reciprocal cross-breeding of Duroc and Berlin Miniature Pig breeds. The pedigree used for analysis included 3 Duroc and 3 Berlin Miniature Pig grandparents, 11 F_1 sows and three F_1 boars and 21 F_2 full sib-families totaling 438 animals. All F_2 animals were kept and performance tested at the research farm Frankenforst of the Institute of Animal Breeding and Genetics, University of Bonn. Piglets were weaned at about 6 weeks of age and kept in flat decks until day 100 and subsequently in single pens until slaughter at 200 days of age. Male piglets were castrated within the first week of life. Overall association of the c.+83G > A SNP with the variation in LMA, BFT, CL, and ADG was tested using analysis of variance (PROC GLM; SAS V8.2). Apart from fixed effects of c.+83G > A genotype the general linear model included fixed effects of sex and F_2 family for all traits, fixed effect of parity for LMA, fixed effect of year season for ADG, and slaughter weight as a covariate for LMA, BFT, and CL. Additive and dominance effects were estimated using linear contrasts (0.5, 0, −0.5 for additive and 0.5, −1, 0.5 for dominance effects) of least square means of c.+83G > A genotypes. Multipoint linkage map of chromosome 4 was established using the BUILD and FLIPS options of CRI-MAP 2.4 package. Genotype information of 5 microsatellite loci (S0227, S0001, S0214, S0097, and STS-Bo4) and of 2 biallelic loci (STS-Bo1 and STS-Bo2) was obtained as described previously [12]. For QTL mapping the sex-averaged recombination fractions were converted to map distances using the Haldane mapping function. The QTL mapping was performed using a regression model for analysis of F_2 crosses [17] implemented in the web-based program package QTL express [18] (http://qtl.cap.ed.ac.uk). This model assumes a biallelic QTL fixed for alternative alleles in each parental line. Based on the marker information probabilities for the inheritance of the four possible QTL genotypes are estimated at 1-cM intervals along the chromosome. These probabilities are used to calculate additive and dominance coefficients for a putative QTL at each position and the trait values are then regressed onto these coefficients. The least square regression model fitted was the same as used in the association analysis with the only exception that additive and dominance QTL effects replaced the c.+83G > A genotype effect. Chromosome-wide significance thresholds were derived empirically by 10,000 permutations and on their basis approximate genome-wide thresholds were derived by Bonferroni correction. The 5% chromosome-wide significance threshold was used as a threshold for suggestive linkage. Confidence intervals for QTL position were estimated by generating 1000 bootstrap resamples. Permutation and bootstrap analysis were performed using QTL express. To evaluate the association of the c.+83G > A SNP genotype with the detected QTL effects, the c.+83G > A SNP genotype was included as a fixed effect in the QTL regression model. If the c.+83G > A SNP is causal or in linkage disequilibrium with the QTL, it is expected to absorb part of the QTL effect and cause a drop in the QTL significance [19].

Inference of haplotypes from genotypic data on g.233 C > T, c.-15 + 8 G > T, c.*44 (A)6-7, and c.*151C > G was performed using the HAPAR software [20].

Results and discussion

Characterization and polymorphisms of the pCRH gene

Starting from the published partial sequence of exon 2 we completed the cDNA sequence of the p*CRH* gene by performing 5′- and 3′-RACE, determined its genomic structure by cloning of the intron, and isolated approximately 600 bp of the 5′-flanking region. The whole sequence obtained spanned 2650 bp (DQ358705). The

Table 2
Primers and amplification conditions used for genotyping and physical mapping

Target	Primer sequence 5′–3′	Size (bp)	Ta (°C)
g.233 C > T	Fwd: ACAGGACTGTGGGTTGGTC Rev: AACGAGCCCTAAAATGGAAG	196	55
c.-15 + 8 G > T	Fwd: AACCCTTGAAAACCCCAGAGAC Rev: GAATCTTGAGGCACAGTCAGC	250	55
c.+83G > A	Fwd: CTGTTGGTGGCTCTCTTGC Rev: GCAGCGGGACTCTTATTGAG	212	55
c.*44(A)6-7	Fwd: ird800AGCAACAGGAAACTGATGGAG Rev: AGAGCAACGCTATGGTACAGAA	125/126	55
c.*151C > G	Fwd: AGCAACAGGAAACTGATGGAG Rev: GTCACCCACCCAAGAGAATG	412	55
IMpRH	Fwd: CGCGCAGCTCACCTACAGA Rev: GAATCTTGAGGCACAGTCAGC	206	66–58

CRH gene comprises two exons, with the shorter first exon containing a nearly complete 5′-untranslated region (UTR) and the second exon containing the remaining part of 5′-UTR, whole coding sequence (cds), and 3′-UTR. Genomic structure of the *CRH* gene has already been elucidated in human, rat, and sheep [21–23]. Software tools for cross-species sequence comparison—phylogenetic footprinting—evolved rapidly in conjunction with sequencing of various metazoan genomes and allow today powerful in silico search for evolutionary conserved, presumably functionally important, sequence elements [24]. In this study, we used different in silico tools to characterize the porcine sequence and its variation, and provide new insights into putative regulatory elements of the *CRH* gene.

The 5′-RACE product terminated at position g.604, i.e., 21 bp downstream of the major transcription start reported for human and sheep *CRH* gene [23,25]. This position may represent an alternative, perhaps minor, and transcriptional start site. However, because the transcription start site and adjacent CAAT and TATA boxes found in human/ovine *CRH* gene are structurally and spatially conserved in the porcine sequence (positions g.583, g.519, and g.553, respectively), we tentatively assign the transcription start of the p*CRH* gene to position g.583. The 3′-RACE revealed two cDNA forms polyadenylated using signals at positions g.2559 (c.*428) and g.2626 (c.*495), respectively. The two transcript forms have a predicted length of 1210 and 1274 bp, respectively. Multiple (up to three) *CRH* transcript forms due to polyadenylation at different sites have been reported also in rat and sheep [22,26]. The intron intervenes the cDNA in the 5′-UTR between position c.-15 and c.-14 (g.748–g.1541). Open-reading frame of the p*CRH* gene spans 576 bp and encodes a prepro-hormone of 191 amino acid residues. Alignment of the deduced porcine amino acid sequence with human, canine, bovine, ovine, rat, and murine (Fig. 1) revealed three regions sharing high homology: the mature peptide showing the highest homology (p.149–p.189), the signal peptide region especially around the cleavage site (p.1–p.29), and a prohormone region of yet unknown function (p.64–p.76).

In order to identify potential regulatory regions of the p*CRH* gene, we performed cross-species comparison aligning porcine with corresponding ovine, canine, human, and rat sequences using MULAN. Six segments of the p*CRH* gene fit our criteria for evolutionary conserved region, out of which four are located in the noncoding sequence and hence represent potential regulatory elements: two in the 5′-flanking region and 5′-UTR (ECR1 and 2), one in the intron (ECR3) and one in the 3′-UTR (ECR6; Table 3 and Fig. 1). As shown in Fig. 2, ECR2 spanning approximately 350 bp proximal to transcription start and 65 bp of 5′-UTR exhibits the highest conservation level exceeding even the conservation level of the cds (ECR4 and 5). Besides the conserved CAAT (g.519) and TATA (g.553) boxes several experimentally determined regulatory elements such as cAMP response elements at position g.458, a negative glucocorticoid response element (nGRE) at position g.303, and a glucocorticoid receptor DNA-footprint at g.381 reside within ECR2 and are completely conserved between all five species aligned [27]. This result

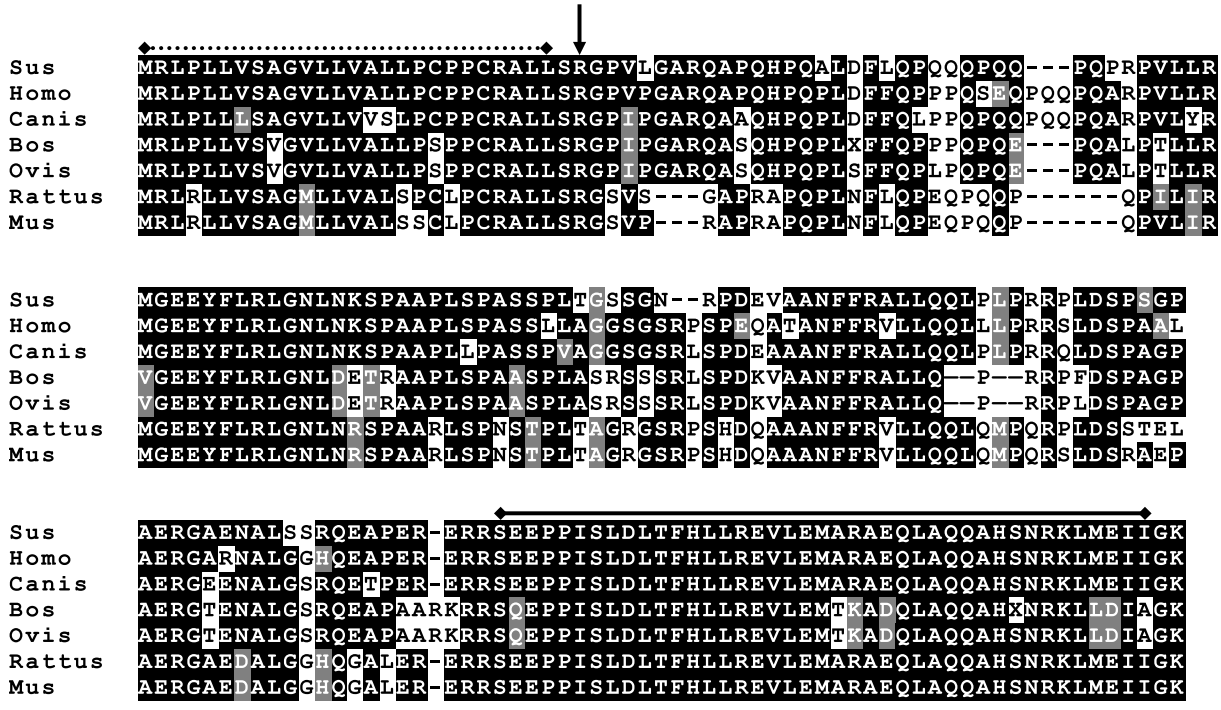

Fig. 1. Comparison of the deduced porcine preproCRH sequence with sequences of other mammalian species. Identical amino acids are highlighted in black and amino acids of the same group in gray. The solid line indicates the mature peptide and the dotted line indicates the signal peptide. The vertical arrow marks the p.28Arg > Gln exchange due to c.+83G > A SNP.

Table 3
Relative positions of evolutionary conserved regions, CpG islands, and polypurine/polypyrimidine elements

Number	ECR	CpG	PPyE
1	g.52–g.109	g.661–g.909	g.175–g.234
2	g.230–g.647	g.1725–g.1995	g.615–g.673
3	g.904–g.991		g.1044–g.1097
4	g.1531–g.1673		g.1484–g.1526
5	g.1729–g.2319		
6	g.2502–g.2641		

demonstrates the usefulness of phylogenetic footprinting to identify regulatory regions in silico.

Transcription of genes is regulated not only through binding of transcription factors (TF) to cognate binding sites and interaction with the general transcription machinery but also through organization of chromatin structure [24]. Mechanisms proposed to modulate chromatin structure and regulate transcription are formation of non-B-DNA conformation (e.g., Z-DNA or H-DNA) and methylation of CpG islands [28,29]. The porcine *CRH* gene harbors two CpG islands: an intronic CpG island spanning nearly the entire sequence between ECR2 and ECR3, and a CpG island in the cds (Table 3 and Fig. 1). The intronic CpG island is preserved at a similar position in all species included, although shorter in canine and rat *CRH* gene (not shown). Methylation of a similarly positioned CpG island in the MCJ gene is associated with cell-type specific expression and modulates the chromatin structure not only of the CpG island itself but also of neighboring regions [30]. Thus, the intronic CpG island may control cell-type specificity of *CRH* gene expression, possibly in cooperation with *cis*-regulatory elements in ECR2. Existence of an intronic element regulating cell-type specific expression of *CRH* gene is supported by the observation of Stenzel-Poore et al. [4] that a *CRH* transgene based on rat sequence where ECR1, ECR2, and ECR6 were replaced with metallothionein-1 gene promoter and 3′-UTR of growth hormone gene, respectively, largely retained the cell-type expression pattern of endogenous *CRH* gene. An additional conserved feature of the *CRH* gene intron is a Z-DNA-forming stretch of alternating purine/pyrimidine residues overlapping with the CpG island. The level of Z-DNA formation by this element in h*CRH* gene correlates with the transcription activity in vitro and was increased by forskolin and decreased by dexamethasone treatment [31]. These findings provide additional evidence that the region including the intronic CpG island participates in chromatin organization and thus in regulation of *CRH* gene expression. Polypurine/polypyrimidine (PPy) elements are capable of forming triple helical H-DNA and are associated with regulation of expression of a number of genes, for example androgen receptor gene [32]. We identified four PPy elements (PPYE1-4) in the available porcine sequence (Table 3). Comparison of pyrimidine content profiles presented in Fig. 3 demonstrates the extent to which the PPy elements are preserved among species. PPYE1 lies in the 5′-flanking region in front of ECR1 and though embedded in nonconserved segment this element is well preserved. PPYE2 is located in exon 1 and overlaps with ECR2. PPYE3 and PPYE4 both lie in the intron in nonconserved segments, with PPYE4 in front of exon 2 and ECR4, and are less well preserved than the two former PPy elements.

The fact, that even though the primary structure of the intronic CpG island, Z-DNA forming element, the PPy elements is variable but the sequence features retained their overall character and are largely preserved among species, indicate, that they are functional and may contribute to species-specific differences in the regulation of the *CRH* gene. This view is supported by Wang and Vasquez [33] who showed that H-DNA elements are mutagenic and proposed that they contribute to genetic evolution.

In total thirteen variable sites were found in the p*CRH* gene sequence (Fig. 2). Similar high level of sequence diversity of transcriptional units in the pig has been reported by Fahrenkrug et al. [34] (1 SNP per 184 bp).

Comparative sequencing of the cds revealed a single G > A SNP at position c.+83. This SNP led to substitution of an arginine residue conserved in mammalian evolution to glutamine at position p.28 (Fig. 2), close to the signal peptide cleavage site at position p.26. In functionally related proopiomelanocortin, the first 26 residues following signal peptide contain a sorting signal directing the prohormone to regulated secretory pathway in neuroendocrine cells [35]. Majewski and Ott [36] predicted that arginine represents one of the least mutable residues. Thus, the p.28Arg>Gln exchange is likely functional and may affect signal peptide cleavage or a sorting signal and affect proCRH secretion. The mutant adenine variant (i.e., Gln variant) originates from Berlin-Miniature Pig. No segregation of this variant in commercial breeds was observed [15].

Comparative sequencing of the 5′-flanking region revealed a C > T SNP at position g.233 on the distal border of ECR2 (Fig. 2). Alignment of a segment harboring the variable g.233 site among nine mammalian species (pig and sheep, cattle, cat, dog, mouse, rat, human, and chimpanzee) revealed that all share the C variant most likely representing the ancestral form. Moreover, a 10-bp "motif" encompassing the SNP site is perfectly conserved among pig, sheep, cattle, cat, mouse, and rat, but distinct in primates (Fig. 4). To identify conserved TFBS affected by the SNP, we performed phylogenetic footprinting aligning pairwise porcine with the other eight mammalian sequences using zPicture/rVista2.0 (Table 4). The g.233 C > T SNP eliminate a glucocorticoid response element (GRE) like half-site as well as binding sites for caudal type homeo box transcription factor 1 (CDX1), cAMP responsive element binding protein (CREB), and signal transducer and activator of transcription (STAT) 1, 4, and 5A (Table 4). Existence of a GRE would be in line with negative feedback inhibition of *CRH* gene transcription in hypothalamus exerted by glucocorticoids [23]. Usually a functional GRE consists of two palindromic half-sites separated by three degenerate nucleotides [37], however GRE

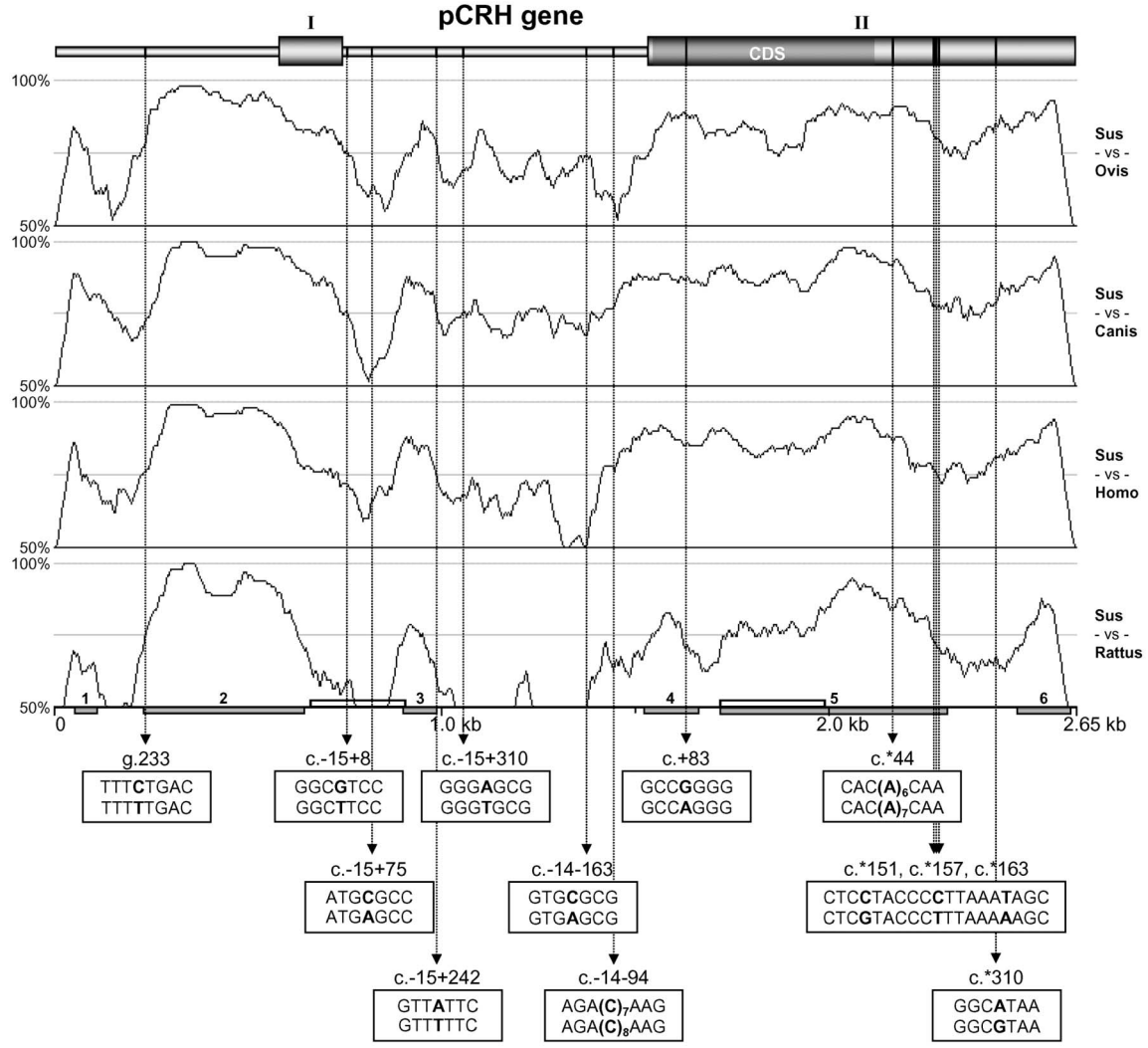

Fig. 2. Evolutionary conservation and variation of the porcine *CRH* gene sequence. At the top is the schematic representation of the p*CRH* gene. Exons are represented as wide cylinders labeled by exon numbers in roman numerals. UTR regions are shown as light gray and the coding sequence as dark gray segments, respectively. The 5′-flanking region and the intron are shown as narrow cylinders. Beneath stacked-pairwise conservation profiles between porcine and ovine, canine, human and rat *CRH* gene sequence generated using Mulan are shown. Numbered gray bars under the *x* axis of the conservation profile represent evolutionary conserved regions (>75% identity; >50 bp), open bars over the *x* axis show CpG islands in the p*CRH* gene. Identified sequence variation of p*CRH* gene is shown at the bottom in open boxes in bold letters.

like half site may also be functional and confer delayed glucocorticoid response as shown by Chan et al. [38]. The occurrence of STAT and CREB binding sites might reflect regulation of *CRH* gene expression by cytokines and protein kinase A pathways, respectively [39]. Which role may CDX1 play in the regulation of *CRH* gene is less obvious. Nicholson et al. [27] recently reported the involvement of a more proximal CDX1 binding site in h*CRH* promoter in cAMP induced stimulation of *CRH* transcription. Thus, the CDX1 binding site at g.233 may function as a cAMP responsive element. To our knowledge functional analysis of *CRH* gene promoter so far revealed no regulatory elements coinciding with the motif encompassing the g.233 site [27]. However, functional studies of *CRH* promoter focused mainly on human sequence, where the motif diverged and the predicted TFBS vanished. Supposing that the porcine motif represents a functional TFBS identification of the cognate TF would be of great interest not only for an understanding of the functional impact of g.233 C > T SNP but also for an understanding of the evolution of primates.

Additional six variable sites were identified in the intron and five in the 3′-UTR (Fig. 2). One SNP in the intron (c.-15 + 242A > T) and four variable sites in the 3′-UTR (c.*44(A)6-7, c.*151C > G, c.*157C > T, and c.*163T > A) lie in ECR. However of these five polymorphisms, only two SNP in ECR6 c.*157C > T and c.*163T > A affect perfectly conserved sites. Phylogenetic footprinting revealed that these two closely spaced SNP affect another CDX1 binding site. No functional studies regarding *cis*-regulatory elements in the intron or 3′-UTR of *CRH* gene were reported up to now. Additional potential regulatory variation in the p*CRH* gene represents SNP c.-15 + 8G > T and c.-15 + 75C > A that eliminate CpG dinucleotides in the

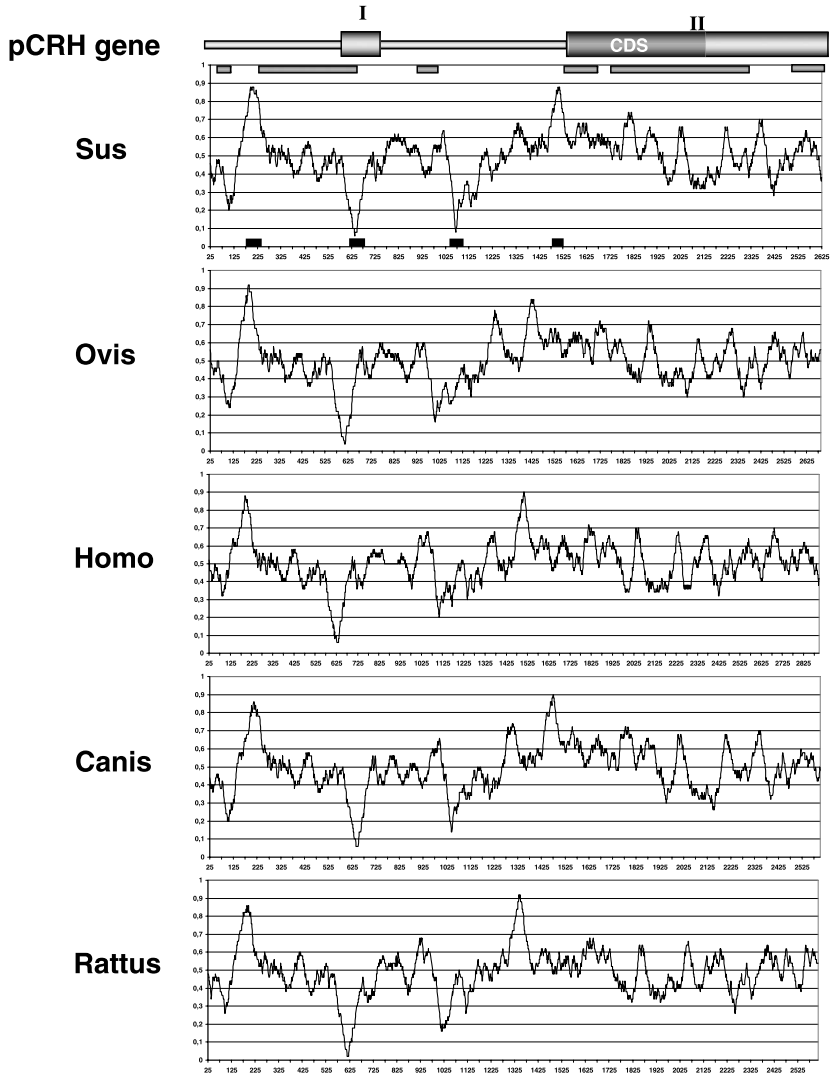

Fig. 3. Pyrimidine content profile of porcine, ovine, human, canine, and rat *CRH* gene. At the top is the schematic representation of the p*CRH* gene with gray bars representing the evolutionary conserved regions. Black boxes on the *x* axis of the porcine profile indicate the position of polypurine/polypyrimidine elements in the p*CRH* gene sequence.

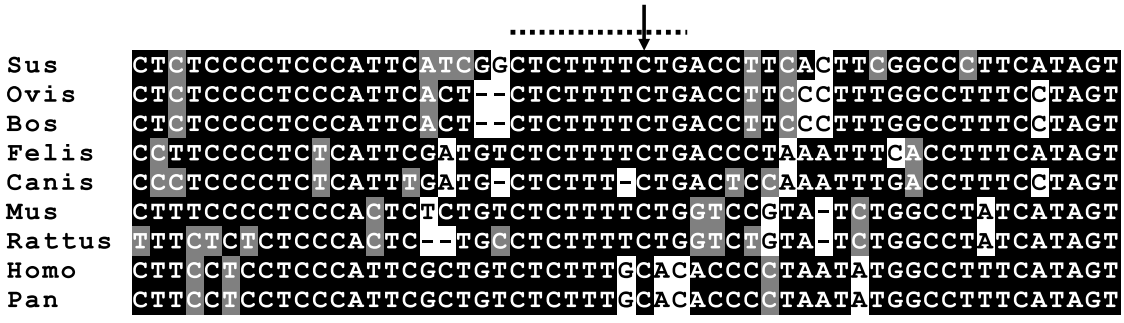

Fig. 4. Multiple alignment of a segment of the 5′-flanking region of *CRH* gene encompassing the g. 233C > T variable site among nine mammalian species. The vertical arrow and the dotted line indicate the position of g. 233C > T SNP and the surrounding conserved motif, respectively.

intronic CpG island and may therefore affect cell-type pattern of p*CRH* gene expression and c.-15 + 310A > T that substitute a purine for a pyrimidine residue on the purine-rich strand of PPYE3 and thus may effect its conformation. Whether any of the presumable regulatory variations is indeed functional remains to be established experimentally.

Sequencing indicated that except the cSNP c.+83G > A all variable sites segregate in commercial breeds. For four variable sites (g.233 C > T, c.-15 + 8G > T, c.*44(A)6-7,

Table 4
List of putative evolutionary conserved transcription factor binding sites affected by DNA-variation in the p*CRH* gene

SNP	TF	TFBS-model	Porcine sequence[a]	Conservation[b]
g.233C > T	GRE	GR_Q6_01	tcTTTT**C**t	Oar, Bta, Fca, Mmu, Rno
	STAT1	STAT1_03	cttTT**c**tg	Oar, Bta, Fca, Mmu, Rno
	STAT4	STAT4_01	cttTT**C**tg	Oar, Bta, Fca, Cfa, Mmu, Rno
	STAT5A	STAT5A_03	cttTT**C**tg	Oar, Bta, Fca, Mmu, Rno
		STAT5A_04	cttTT**C**tg	Oar, Bta, Fca, Cfa, Mmu, Rno
	CDX1	CDXA_02	ttTt**c**tg	Oar, Bta, Fca, Cfa, Mmu, Rno
	CREB	CREB_Q3	**c**TGACC	Oar, Bta, Fca
c.*157C > T/ c.*163T > A	CDX1	CDXA_01	**c**ttaAA**t**	Oar, Bta, Fca, Cfa, Mmu, Rno
	CDX1	CDXA_02	**c**ttaAa**t**	Oar, Bta, Fca, Cfa, Mmu, Rno

[a] Bold letter, variable nucleotides; uppercase letters, TFBS core sequence.
[b] Species where the affected porcine TFBS is either conserved or aligned; Oar, *Ovis aries*; Bta, *Bos taurus*; Fca, *Felis catus*; Cfa, *Canis familiaris*; Mmu, *Mus musculus*; and Rno, *Rattus norvegicus*.

and c.*151C > G) segregation in German Landrace, Large White, Pietrain, and Duroc breeds was confirmed by genotyping (Fig. 5 and Table 5). This four sites form three haplotypes: C-G-A6-C, C-G-A7-G, and T-T-A6-C. Whereas, the individual sites segregate at similar intermediate frequencies in all four commercial breeds, the haplotype frequencies show distinct differences. No segregation of the haplotype C-G-A6-C could be observed in German Landrace and Duroc. In the Wild Pig, the haplotype T-T-A6-C, which is frequent in the commercial pigs, seems to be absent. These results indicate that pig breeding increased frequency of the haplotype T-T-A6-C at the expense of the C-G-A6-C haplotype, which may be associated with an undesirable phenotype that is subject of negative selection.

Association of pCRH gene variation with body composition and growth

As a first step towards dissection of the role of *CRH* gene in the control of traits related to body composition and growth in the pig, we evaluated the p*CRH* gene as a positional candidate for QTL affecting LMA, BFT, CL, and ADG in the DUMI resource population (Table 6). QTL analyses in crosses between divergent pig breeds are based on the assumption that QTL underlying traits for

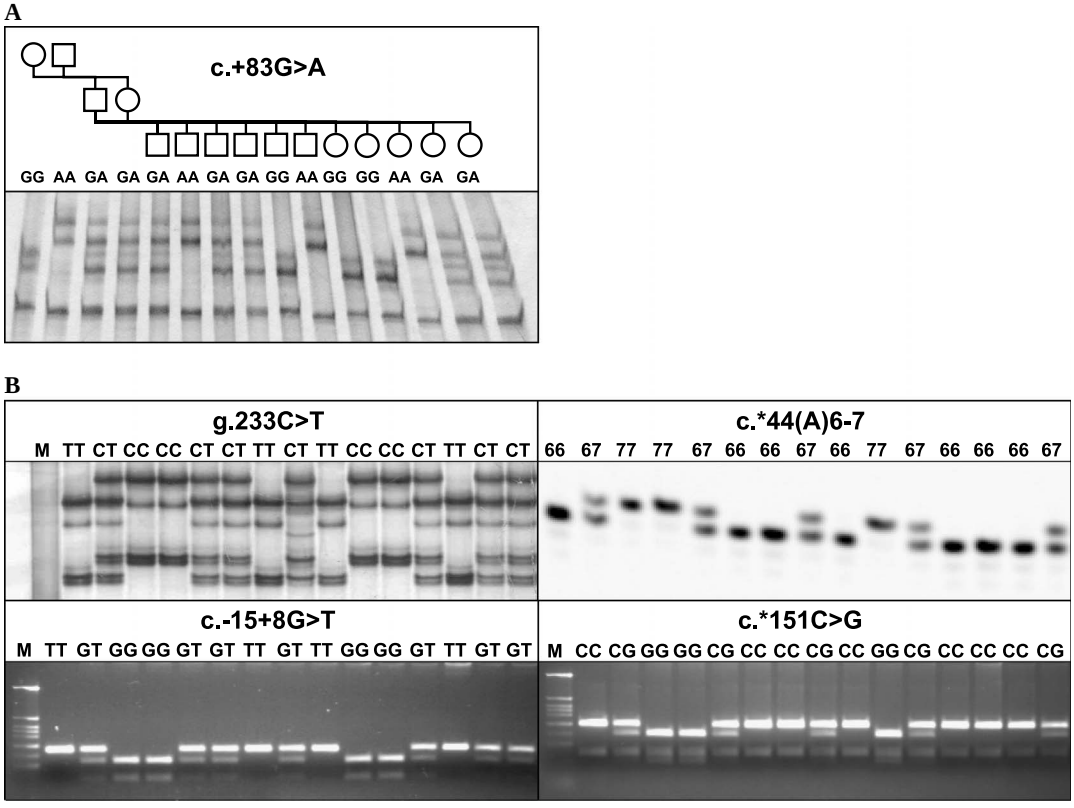

Fig. 5. Segregation of five variable sites in the p*CRH* gene. (A) Mendelian segregation of the c.+83G > A SNP in a three-generation pedigree of the DUMI population. (B) Segregation of g.233C > T, c.-15 + 8G > T, c.*44(A)6-7, and c.*151C > G in each five German Landrace, Large White, and Pietrain commercial pigs.

Table 5
Allele frequencies of analyzed variable sites and their haplotypes in six pig breeds

	LR ($n = 15$)	LW ($n = 15$)	PI ($n = 15$)	DU ($n = 5$)	WP ($n = 2$)	MI ($n = 1$)
g.233 C	0.57	0.53	0.4	0.5	1	0.5
c.-15 + 8 G	0.57	0.53	0.4	0.5	1	0.5
c.*44 (A)6	0.43	0.7	0.83	0.5	0.25	0.5
c.*151C	0.43	0.7	0.83	0.5	0.25	0.5
C-G-A6-C	0	0.1	0.23	0	0.25	0
C-G-A7-G	0.57	0.43	0.17	0.5	0.75	0.5
T-T-A6-C	0.43	0.47	0.6	0.5	0	0.5

LR, German Landrace; LW, Large White; PI, Pietrain; DU, Duroc; WP, Wild pig; and MI, Berlin Miniature Pig.

Table 6
Descriptive statistics of the traits analyzed

Trait	Trait	No.	Mean	SD
Longissimus muscle area (cm^2)	LMA	438	24.42	4.56
Carcass length (cm)	CL	438	81.23	4.47
Average backfat thickness (cm)	BFT	438	4.44	0.89
Average daily gain (g/day)	ADG	438	533.73	96.00

which the crossed lines diverge are largely fixed [17]. Of the 13 variable sites found in the p*CRH* gene the c.+83G>A SNP fits the assumption best. The G variant is fixed in commercial breeds and also in the Duroc grandparents of DUMI population [15]. Two Miniature pig grandparents were homozygous for the A variant and one was found to be heterozygous. As the A variant likely affects the secretion of p*CRH* the c.+83G > A SNP was also functionally most relevant. A defect in the synthesis or release of CRH has been implicated in the development of obesity in laboratory animals [40].

In a previous study, we showed that the marker interval STS-Bo3–STS-Bo1 harboring the QTL most likely corresponds in inverse order with the region q12.3–q21.13 of human chromosome 8 (more precisely interval 64.71–79.26 Mbp) encompassing the *CRH* gene on q13 at 67.25 Mbp [12]. Using the c.+83G > A SNP information we genetically mapped the p*CRH* gene in the interval between STS-Bo3 and STS-Bo1 in line with the prediction based on the comparative evidence. The map distance in cM in the pig (STS-Bo3–4.5 cM–CRH–1.1 cM–STS-Bo1; sex-averaged distances in Haldane cM) is directly proportional to physical distance in Mbp in humans (Fig. 6). Our comparative mapping of the linkage group STS-Bo3–CRH–STS-Bo1 is in good agreement with the current high-resolution comparative map of Ssc4 (http://www.animalgenome.org/cgi-bin/QTLdb/viewmap).

After inclusion of the p*CRH* gene in the linkage map, we reran the QTL analysis. As shown in Fig. 6 and Table 7 the position of p*CRH* at 80.8 cM coincides with the position of QTL for ADG and falls 1 cM apart and in the confidence interval of QTL affecting LMA, BFT, and CL. This result promotes the p*CRH* gene as a positional candidate. Already the first genome scan in the pig performed in a Wild Boar × Large White F_2-cross revealed large QTL effects on body composition and growth in central part of the porcine chromosome 4 [41]. QTL affecting body composition and growth in this region were repeatedly found in various intercrosses of divergent porcine breeds [42,43]. Unfortunately the markers STS-Bo3, STS-Bo1,

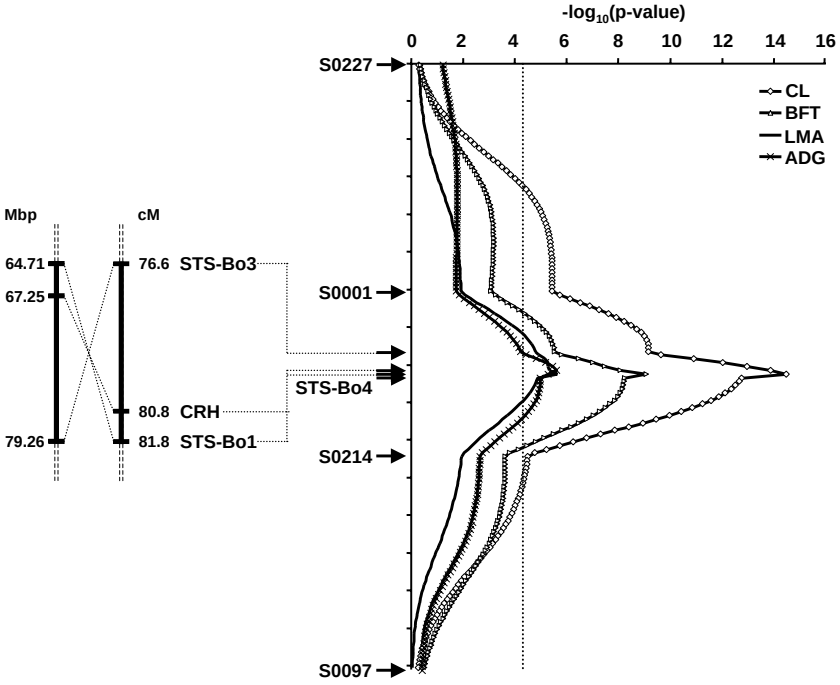

Fig. 6. QTL affecting body composition and growth mapped on Ssc4 in the DUMI population and comparative mapping of the QTL region between Hsa8 sequence map (Hsa build 35.1) and the Ssc4 linkage map.

Table 7
Significance, position, and effects of QTL on Ssc4 in the DUMI population and their significance and position after inclusion of the c.+83G > A genotype

Trait	$-\log_{10}$ (p value)[a]	Position (CI)	Additive effect (SE)	Dominance effect (SE)	$-\log_{10}$ (p value)[a,b]	Position[b]
LMA (cm^2)	5.65**	82 (70–91.5)	1.44 (0.28)	0.61 (0.39)	3.26†	82
CL (cm)	14.48**	82 (81–82)	1.90 (0.22)	−0.13 (0.30)	3.04†	82
BFT (cm)	9.08**	82 (81–89)	−0.33 (0.05)	0.05 (0.07)	3.75*	82
ADG (g/day)	5.63**	81 (10.5–94.5)	32.97 (6.56)	−9.38 (9.34)	2.58†	81

[a] $-\log_{10}$ (p value) with superscripts **, * are significant at the genome-wide 1% or genome-wide 5%, respectively; superscript † indicates suggestive linkage.
[b] Significance and position of QTL after inclusion of the c.+83G > A genotype.

Table 8
Overall association of the c.+83G > A SNP in the p*CRH* gene with body composition and growth in the DUMI population

Trait	$-\log_{10}$ (p value)	GG (SE)	GA (SE)	AA (SE)	Additive effect (SE)	Dominance effect (SE)
LMA (cm^2)	3.33	25.07 (0.42)	24.22 (0.37)	22.64 (0.53)	1.21[a] (0.31)	−0.36 (0.39)
CL (cm)	11.69	82.82 (0.30)	80.78 (0.24)	79.32 (0.39)	1.75[a] (0.24)	0.29 (0.31)
BFT (cm)	6.09	4.18 (0.07)	4.52 (0.06)	4.74 (0.09)	−0.28[a] (0.06)	−0.06 (0.07)
ADG (g/day)	3.99	554.87 (9.37)	525.05 (8.20)	494.10 (11.96)	30.39[a] (7.11)	−0.56 (9.46)

[a] Nominal significant at $p < 0.0001$.

and STS-Bo4 bracketing the QTL and p*CRH* gene are specific to our population, which complicates comparison of our mapping results with other studies. Thus, we used the INRA-University of Minnessota radiation hybrid (IMpRH) panel to anchor the p*CRH* gene on current consensus physical and linkage maps of Ssc4. We assigned p*CRH* into marker interval SW724-S0107 (SW724–0.82 cR–CRH–0.53 cR–S0107) again in accord with the current comparative map of Ssc4. This location points to the p*CRH* gene as a positional candidate also for fatness and growth QTL mapped by Knott et al. [44], and Bidanel et al. [42] and for QTL affecting muscularity and carcass length mapped by Varona et al. [43].

Two additional statistic analyses were performed to test whether the QTL effects are associated with the c.+83G > A SNP. First overall association of p*CRH* gene with LMA, BFT, CL, and ADG was tested by fitting c.+83G > A SNP as a fixed effect in a general linear model. For all traits the c.+83G > A SNP has a significant additive effect (Table 8). In accord with direction of the QTL effects and with the phenotypic differences between the breeds, the A allele, inherited from Miniature pig, decreases lean mass and growth. The magnitude and significance of the effects of c.+83G > A SNP are slightly lower than those of the QTL alleles. In the subsequent analysis, the c.+83G > A genotype was included as a fixed effect in the QTL regression analysis. If the c.+83G > A SNP is causal or in linkage disequilibrium with the QTL, it is expected to absorb part of the QTL effect and cause a drop in the QTL significance [19]. The inclusion of the c.+83G > A genotype caused a large drop in the QTL significance, comparable with the fixed effect of c.+83G > A SNP in the general linear model, however for none of the traits the evidence for a QTL was completely eliminated. This result indicates that the c.+83G > A SNP might be involved in the observed QTL effects but there is/are probably another QTN underlying the QTL effects in addition to c.+83G > A SNP. Several studies point to multiple QTL in the central part of Ssc4 [45,46]. In the mouse, there are an increasing number of QTL studies where large QTL when fine-mapped turned out to be due to multiple linked loci [47]. However given the close proximity between p*CRH* gene to the estimated QTL positions in the DUMI population and the nearly complete fixation of c.+83G > A SNP in parental breeds, the association of c.+83G > A SNP and LMA, BFT, CL, ADG, and corresponding QTL effects may be merely due to linkage disequilibrium. Simulation studies show that it is generally difficult to discriminate between the effect of a QTN and of another SNP when both are located in the same region and the analyzed SNP is fixed or nearly fixed in the parental populations [19]. The knowledge of DNA-variation of the p*CRH* gene provided in this study will facilitate follow-up studies of additional, most suitably closed, mating populations [19] that is required to prove the effect of p*CRH* gene on body composition and growth.

References

[1] G.P. Chrousos, Stressors, stress, and neuroendocrine integration of the adaptive response. The 1997 Hans Selye Memorial Lecture, Ann. N Y Acad. Sci. 851 (1998) 311–335.
[2] E.J. Squires, Applied Animal Endocrinology, CABI Publishing, 2003.
[3] A. Foury, T. Devillers, M.P. Sanchez, H. Griffon, P. Le Roy, P. Mormede, Stress hormones, carcass composition and meat quality in Large White × Duroc pigs, Meat Sci. 69 (2005) 703–707.
[4] M.P. Stenzel-Poore, V.A. Cameron, J. Vaughan, P.E. Sawchenko, W. Vale, Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice, Endocrinology 130 (1992) 3378–3386.
[5] F. Rohner-Jeanrenaud, C.D. Walker, R. Greco-Perotto, B. Jeanrenaud, Central corticotropin-releasing factor administration prevents the excessive body weight gain of genetically obese (fa/fa) rats, Endocrinology 124 (1989) 733–739.

[6] R.F. Parrott, Central administration of corticotropin releasing factor in the pig: effects on operant feeding, drinking and plasma cortisol, Physiol. Behav. 47 (1990) 519–524.
[7] X.Y. Lu, G.S. Barsh, H. Akil, S.J. Watson, Interaction between alpha-melanocyte-stimulating hormone and corticotropin-releasing hormone in the regulation of feeding and hypothalamo–pituitary–adrenal responses, J. Neurosci. 23 (2003) 7863–7872.
[8] T. Masaki, G. Yoshimichi, S. Chiba, T. Yasuda, H. Noguchi, T. Kakuma, T. Sakata, H. Yoshimatsu, Corticotropin-releasing hormone-mediated pathway of leptin to regulate feeding, adiposity, and uncoupling protein expression in mice, Endocrinology 144 (2003) 3547–3554.
[9] L. Perusse, T. Rice, Y.C. Chagnon, J.P. Despres, S. Lemieux, S. Roy, M. Lacaille, M.A. Ho-Kim, M. Chagnon, M.A. Province, D.C. Rao, C. Bouchard, A genome-wide scan for abdominal fat assessed by computed tomography in the Quebec Family Study, Diabetes 50 (2001) 614–621.
[10] F.C. Buchanan, T.D. Thue, P. Yu, D.C. Winkelman-Sim, Single nucleotide polymorphisms in the corticotrophin-releasing hormone and pro-opiomelancortin genes are associated with growth and carcass yield in beef cattle, Anim. Genet. 36 (2005) 127–131.
[11] E. Murani, K. Wimmers, S. Ponsuksili, K. Schellander, A QTL for eye muscle area on porcine chromosome 4 detected by means of AFLP selective genotyping, in: Proceedings of the Seventh World Congress on Genetics Applied to Livestock Production, 2002, pp. 3–12.
[12] K. Wimmers, E. Murani, S. Ponsuksili, M. Yerle, K. Schellander, Detection of quantitative trait loci for carcass traits in the pig by using AFLP, Mamm. Genome 13 (2002) 206–210.
[13] I. Ovcharenko, G.G. Loots, B.M. Giardine, M. Hou, J. Ma, R.C. Hardison, L. Stubbs, W. Miller, Mulan: multiple-sequence local alignment and visualization for studying function and evolution, Genome Res. 15 (2005) 184–194.
[14] G.G. Loots, I. Ovcharenko, L. Pachter, I. Dubchak, E.M. Rubin, rVista for comparative sequence-based discovery of functional transcription factor binding sites, Genome Res. 12 (2002) 832–839.
[15] K. Wimmers, E. Murani, S. Ponsuksili, K. Schellander, Sequence variation and linkage mapping of the porcine corticotropin releasing hormone (CRH) gene, Anim. Genet. 33 (2002) 233–234.
[16] M. Yerle, P. Pinton, A. Robic, A. Alfonso, Y. Palvadeau, C. Delcros, R. Hawken, L. Alexander, C. Beattie, L. Schook, D. Milan, J. Gellin, Construction of a whole-genome radiation hybrid panel for high-resolution gene mapping in pigs, Cytogenet. Cell Genet. 82 (1998) 182–188.
[17] C.S. Haley, S.A. Knott, J.M. Elsen, Mapping quantitative trait loci in crosses between outbred lines using least squares, Genetics 136 (1994) 1195–1207.
[18] G. Seaton, C.S. Haley, S.A. Knott, M. Kearsey, P.M. Visscher, QTL express: mapping quantitative trait loci in simple and complex pedigrees, Bioinformatics 18 (2002) 339–340.
[19] H. Zhao, M.F. Rothschild, R.L. Fernando, J.C. Dekkers, Tests of candidate genes in breed cross populations for QTL mapping in livestock, Mamm. Genome 14 (2003) 472–482.
[20] L. Wang, Y. Xu, Haplotype inference by maximum parsimony, Bioinformatics 19 (2003) 1773–1780.
[21] S. Shibahara, Y. Morimoto, Y. Furutani, M. Notake, H. Takahashi, S. Shimizu, S. Horikawa, S. Numa, Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene, EMBO J. 2 (1983) 775–779.
[22] R.C. Thompson, A.F. Seasholtz, J.O. Douglass, E. Herbert, The rat corticotropin-releasing hormone gene, Ann. N Y Acad. Sci. 512 (1987) 1–11.
[23] P.J. Roche, R.J. Crawford, R.T. Fernley, G.W. Tregear, J.P. Coghlan, Nucleotide sequence of the gene coding for ovine corticotropin-releasing factor and regulation of its mRNA levels by glucocorticoids, Gene 71 (1988) 421–431.
[24] W.W. Wasserman, A. Sandelin, Applied bioinformatics for the identification of regulatory elements, Nat. Rev. Genet. 5 (2004) 276–287.
[25] D.M. Frim, R.L. Emanuel, B.G. Robinson, C.M. Smas, G.K. Adler, J.A. Majzoub, Characterization and gestational regulation of corticotropin-releasing hormone messenger RNA in human placenta, J. Clin. Invest. 82 (1988) 287–292.
[26] Y. Furutani, Y. Morimoto, S. Shibahara, M. Noda, H. Takahashi, T. Hirose, M. Asai, S. Inayama, H. Hayashida, T. Miyata, S. Numa, Cloning and sequence analysis of cDNA for ovine corticotropin-releasing factor precursor, Nature 301 (1983) 537–540.
[27] R.C. Nicholson, B.R. King, R. Smith, Complex regulatory interactions control CRH gene expression, Front. Biosci. 9 (2004) 32–39.
[28] K. van Holde, J. Zlatanova, Unusual DNA structures, chromatin and transcription, Bioessays 16 (1994) 59–68.
[29] L. Lande-Diner, H. Cedar, Silence of the genes—mechanisms of long-term repression, Nat. Rev. Genet. 6 (2005) 648–654.
[30] G. Strathdee, B.R. Davies, J.K. Vass, N. Siddiqui, R. Brown, Cell type-specific methylation of an intronic CpG island controls expression of the MCJ gene, Carcinogenesis 25 (2004) 693–701.
[31] S. Wolfl, C. Martinez, A. Rich, J.A. Majzoub, Transcription of the human corticotropin-releasing hormone gene in NPLC cells is correlated with Z-DNA formation, Proc. Natl. Acad. Sci. USA 93 (1996) 3664–3668.
[32] S. Chen, P.C. Supakar, R.L. Vellanoweth, C.S. Song, B. Chatterjee, A.K. Roy, Functional role of a conformationally flexible homopurine/homopyrimidine domain of the androgen receptor gene promoter interacting with Sp1 and a pyrimidine single strand DNA-binding protein, Mol. Endocrinol. 11 (1997) 3–15.
[33] G. Wang, K.M. Vasquez, Naturally occurring H-DNA-forming sequences are mutagenic in mammalian cells, Proc. Natl. Acad. Sci. USA 101 (2004) 13448–13453.
[34] S.C. Fahrenkrug, B.A. Freking, T.P. Smith, G.A. Rohrer, J.W. Keele, Single nucleotide polymorphism (SNP) discovery in porcine expressed genes, Anim. Genet. 33 (2002) 186–195.
[35] D.R. Cool, M. Fenger, C.R. Snell, Y.P. Loh, Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway, J. Biol. Chem. 270 (1995) 8723–8729.
[36] J. Majewski, J. Ott, Amino acid substitutions in the human genome: evolutionary implications of single nucleotide polymorphisms, Gene 305 (2003) 167–173.
[37] M. Beato, Transcriptional control by nuclear receptors, FASEB J. 5 (1991) 2044–2051.
[38] G.C. Chan, P. Hess, T. Meenakshi, J. Carlstedt-Duke, J.A. Gustafsson, F. Payvar, Delayed secondary glucocorticoid response elements. Unusual nucleotide motifs specify glucocorticoid receptor binding to transcribed regions of alpha 2u-globulin DNA, J. Biol. Chem. 266 (1991) 22634–22644.
[39] M. Pisarska, J.J. Mulchahey, S. Sheriff, T.D. Geracioti, J.W. Kasckow, Regulation of corticotropin-releasing hormone in vitro, Peptides 22 (2001) 705–712.
[40] N.J. Rothwell, Central effects of CRF on metabolism and energy balance, Neurosci. Biobehav. Rev. 14 (1990) 263–271.
[41] L. Andersson, C.S. Haley, H. Ellegren, S.A. Knott, M. Johansson, K. Andersson, L. Anderssoneklund, I. Edforslilja, M. Fredholm, I. Hansson, J. Hakansson, K. Lundstrom, Genetic-mapping of quantitative trait loci for growth and fatness in pigs, Science 263 (1994) 1771–1774.
[42] J.P. Bidanel, D. Milan, N. Iannuccelli, Y. Amigues, M.Y. Boscher, F. Bourgeois, J.C. Caritez, J. Gruand, P. Le Roy, H. Lagant, R. Quintanilla, C. Renard, J. Gellin, L. Ollivier, C. Chevalet, Detection of quantitative trait loci for growth and fatness in pigs, Genet. Sel. Evol. 33 (2001) 289–309.
[43] L. Varona, C. Ovilo, A. Clop, J.L. Noguera, M. Perez-Enciso, A. Coll, J.M. Folch, C. Barragan, M.A. Toro, D. Babot, A. Sanchez, QTL mapping for growth and carcass traits in an Iberian by Landrace pig intercross: additive, dominant and epistatic effects, Genet. Res. 80 (2002) 145–154.
[44] S.A. Knott, L. Marklund, C.S. Haley, K. Andersson, W. Davies, H. Ellegren, M. Fredholm, I. Hansson, B. Hoyheim, K. Lundstrom, M. Moller, L. Andersson, Multiple marker mapping of quantitative trait

loci in a cross between outbred wild boar and large white pigs, Genetics 149 (1998) 1069–1080.
[45] S.A. Knott, P.E. Nystrom, L. Andersson-Eklund, S. Stern, L. Marklund, L. Andersson, C.S. Haley, Approaches to interval mapping of QTL in a multigeneration pedigree: the example of porcine chromosome 4, Anim. Genet. 33 (2002) 26–32.
[46] A. Mercade, J. Estelle, J.L. Noguera, J.M. Folch, L. Varona, L. Silio, A. Sanchez, M. Perez-Enciso, On growth, fatness, and form: a further look at porcine chromosome 4 in an Iberian × Landrace cross, Mamm. Genome 16 (2005) 374–382.
[47] J. Flint, W. Valdar, S. Shifman, R. Mott, Strategies for mapping and cloning quantitative trait genes in rodents, Nat. Rev. Genet. 6 (2005) 271–286.